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Investigation of the anti-bacterial properties of mangrove fern, *Acrostichum aureum* in the Niger Delta, Nigeria

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Mangroves render several ecosystem services globally, one of which is pharmacological products. This study was done to verify whether *Acrostichum aureum*, a species of mangrove, common in the Niger Delta has anti-bacterial property. Several leaf samples were plucked from the trees at Eagle Island and placed in a cooler and sent to the lab for further analysis. The leaves were dried and ground into fine powder with manual grinding machine and 100 g of the powdered sample was measured and placed in 1000 ml of each of the extraction solvents (hot water and methanol). Five different concentrations (32.25 mg/ml, 65.5 mg/ml, 125 mg/l, 250 mg/l and 500 mg/l) were made from each of the extracts using dimethyl sulfoxide (DMSO). Furthermore, bacterial species (*Escherichia coli*, *Salmonella paratyphi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) were isolated and a stock of each sample was made in agar slant and stored in the refrigerator at 4°C. Mueller Hinton Sensitivity Agar (MHA) medium was prepared in agar and triplicate discs of each of the concentrations made from the two extracts were placed on the medium (MHA). The zone of inhibition in diameter in all plates was measured and analyzed statistically using R statistics. The result revealed that there is a significant difference in the growth of the microbes on the different concentrations of *A. aureum* ($F_{4, 100} = 4.02$, $P = 0.01$). *A. aureum* had higher effect on *E. coli* and *S. aureus*. Similarly, the higher the concentrations of the extracts the more effective it is in controlling the bacteria. Finally, the study revealed that *A. aureum* has antibacterial properties that can be employed in drug production to treat common diseases prevalent in the region.

Key words: Antibiotics, bacteria, Niger Delta, mangrove, microbial species, medicinal products.

INTRODUCTION

Mangroves are herbaceous plants that grow in coastal areas in the tropical zones of the world. They are found in several countries such as Indonesia, China, Bangladesh, United States (Florida), and Australia (Kathiresan and Bingham, 2001). In Africa, mangroves are found in Nigeria, Cameroun, Ghana, and most West and Central African countries (Spalding et al., 2010; Adams and Rajkaran, 2021). However, the mangroves of Nigeria are the largest in Africa and the third largest in the world (Numbere, 2018a). Nigeria's mangroves are concentrated

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in the southern part of the country called the Niger Delta. Although, the population of mangroves are declining because of deforestation caused by exploration and urbanization activities and use of their stems for firewood. There are over hundred species of mangroves globally, but in the Niger Delta region the species commonly found are red (*Rhizophora* species), black (*Avicennia germinans*), white (*Laguncularia racemosa*), and golden leather fern (*Acrostichum aureum*) mangroves (Numbere and Camilo, 2018). Mangrove ferns are often found in sandy soil away from the river edge towards upland areas or areas with low river tidal height. They usually grow in few bunches of forest stands that are not greater than 1.8 m tall and 3-4 m wide par stand. They are also indicators of a recovering site. Mangroves provide numerous ecosystem services that are useful to humans and the environment such as air purification, food, aquaculture, and medicine. Mangrove parts have pharmacological properties that are of medical importance. For instance, a species of black mangrove twigs found in Asia (*Avicennia marina*) has antimicrobial properties against *Mycobacterium* species, *Staphylococcus aureus* and *Candida albicans* (Han et al., 2007). Similarly, leaf extracts from *A. marina* displayed antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Salmonella aureus* (Devi et al., 2015). *A. marina*, a mangrove species, has cytotoxic activity against cancer cells (Eswaraiah et al., 2020). Other properties include mosquito repellence, anti-bacteriophage, anti-viral, anti-arthritis and antinociceptive properties. Extracts from the leaves and roots of *A*. *aureum* is an antioxidant, and has antibacterial and anti-inflammatory properties (Thomas, 2012). It is also shown that *A. aureum* can be used as an analgesic (that is, pain treatment) when ethanol extract of its products was used on mice (Khan et al., 2013). It has also been demonstrated that extracts of *A. aureum* has inhibitory effect on cancer cells (Dai et al., 2005; Uddin et al., 2011). Further studies had shown that *A. aureum* has wound healing and anti-diarrheal qualities. Similarly, *A. aureum* is shown to have antiviral and anti-parasitic properties (Devi et al., 2015). They also have wound healing properties when liquid from their leaves is squeezed on the surface of open wounds; this was tested on rats by Kimura et al. (2017).

A. aureum is a mangrove species found in many locations across the Niger Delta, but because of the ignorance in its pharmacological properties only few studies had been conducted to test its medicinal properties (Numbere, 2018b). Field observations show that their population is declining rapidly because of deforestation activities such as sand mining, urbanization, and oil exploration (Numbere, 2021). It is thus, important to investigate the potentials of developing drugs from the parts of this species to solve public health problems. The major purpose of the study is to determine whether *A. aureum* extract has anti-bacterial property.

This study is conducted to fill the data gap on the use of mangrove parts for the manufacture of medicinal products in the Niger Delta. This is because there are limited studies done to test the anti-bacterial properties of the mangrove species, *A. aureum.* The study will thus provide data for future studies that will be helpful in the conversion of mangrove parts into medicinal drugs. The objective of this study, therefore, includes the following: (i) to determine if different concentrations of *A. aureum* extract influence the zone of inhibition (diameter) of bacterial species, (ii) to determine if there is a significant difference in the zone of inhibition, and (iii) to determine if different solvents (that is, water and methanol) influence the zone of inhibition of bacterial species.

MATERIALS AND METHODS

Description of study area

The leaf samples were collected from a section of a deforested and sand filled mangrove forest at Eagle Island in the Niger Delta (N04°47; E006°58) and sent to the laboratory for identification and processing. The location is a dense mangrove forest that was cut and later used as a dumping ground for dredged sand. The area experiences rainfall in every month of the year with an annual mean of 3567.4 mm (Gobo, 2001). The mean monthly temperature ranges from 28 to 34°C. The adjoining river is an estuary with a salinity range of 1.45 to 1.62%. The soil is sandy to muddy (swampy) and grades from white to dark brown in color. The soil pH ranges from 6 to 7. *A. aureum* grow at the sandy side of the forest or shallow river zone.

Description of bacterial species

The shape and activities of the different bacteria species used for the study are shown in the following.

E. coli

It is a large and diverse group of bacteria species that are Gram negative. It is a facultative anaerobe that is rod-shaped (Eckburg et al., 2005; Tenaillon et al., 2010). It is usually harmless, but pathogenic strains can cause illnesses (Vergara et al., 2020). It causes diarrhea, urinary tract infections, pneumonia, and respiratory illnesses. *E. coli* is a common species that is found almost everywhere such as meat, vegetable, and other food sources; it is also part of the normal gut flora and found predominantly in fecal matter.

Salmonella paratyphi

This is a genus of rod-shaped (bacillus) Gram negative, and of the family Enterobacteriaceae. It is non-spore forming and motile. It is a facultative anaerobe which produces ATP with oxygen (Fàbrega and Vila, 2013). *S. paratyphi* causes typhoid fever (Ryan and Ray, 2004).

Staphylococcus aureus

This is a Gram-positive cocci (round-shaped) bacterium. It is a

member of the microbiota of the body, mostly found in the upper respiratory tract and on the skin (Todar, 2010). It is a facultative anaerobe that grows without the presence of oxygen (Masalha et al., 2001). Humans are regarded as carriers of *S. aureus* (Tong et al., 2015). It causes skin infections, respiratory diseases, and food poisoning (Todar, 2010).

P. aeruginosa

This is an encapsulated Gram-negative and rod-shaped bacterium that can cause disease in plant, animal and humans. It has strong anti-biotic resistance ability and ubiquitous in the environment. It is an opportunistic organism that invades during a preexisting disease condition such as during cystic fibrosis disease. It can colonize critical part of the body such as the lungs, urinary tract, and the kidney (Wagner et al., 2016).

Collection and preparation of plant sample

Fresh leaf samples of *A. aureum* were collected in a mangrove forest at Eagle Island in April 2020. The leaves were sent to the laboratory and identified by a plant taxonomist. Large quantities of the fresh leaves were air-dried without exposure to direct sun light. The dried leaves of *A. aureum* were cut into small pieces and ground with manual grinder (Corona). The powder of the plant sample was packed in small transparent polyethylene bags and stored in cool, dry cupboard for further use.

Extraction procedure

The powdered leaf sample was extracted using hot water and methanol solvents. Methanol solvents were used because it is bipolar and dissolve in lipids. It is widely used in studies for drug purification (Zhao et al., 2020) and formation of solvate (Yuan et al., 2020), whereas water is a universal solvent that is generally used in the laboratory. The protocol proposed by Mikayel et al. (2017) for the extraction of medicinal plant was followed. In each extraction process, 100 g of dry powder sample of *A. aureum* leaves was soaked in 1000 ml of each solvent with ratio of 1:10 (W/V) of the plant material to solvents. The mixtures were extracted up on shaking with DREHZAHL electronic magnetic stirrer (IKAMAG REO 79219 Staufen, Germany) for 48 h. The extract mixture from the hot water (100°C) was filtered and concentrated by heating the filtrate on a hot plate while the filtrate from the methanol extract was kept standing at room temperature (26°C) for 3 days to eliminate the extraction solvent. Extracts of hot water and methanol yielded 1.8 g (1.8%) and 2.0 g (2.0%), respectively. This occurred after the removal of the extraction solvents and leaf shaft.

Preparation of sensitivity discs from plant extracts

The disc diffusion method was used for this study. With the aid of a micropipette, five different concentrations of the plant extract solutions (500, 250, 125, 62.5 and 31.25) and one control solution using water were prepared with a concentrated solution of Dimethyl sulfoxide (DMSO) for each of the extracts. Sterile discs made using Whatman No. 1 Filter Paper, of 8-mm diameter were embedded with 20 μl/disc of the various concentrations made from plant extracts. The discs were dried in the incubator at 40°C.

Isolation of microorganisms

The test bacteria used for this study were isolated from patients' samples in a medical diagnostic laboratory (Cheesbrough, 2000). The characterization of these bacteria was done using culture dependent techniques and biochemical tests results (Pelczer et al., 2003). The culture dependent technique was used for this study because it is less expensive as compared to the molecular technique. The culture technique is widely used for comparing and isolating individual microorganisms, which are further identified using morphological features, microscopy, and biochemical tests. The four bacteria characterized and used were *E. coli*, *S. paratyphi*, *S. aureus*, and *P. aeruginosa*. The isolated microorganisms were sub-cultured on nutrient agar slants, incubated at 37°C for 24 h and stored in a refrigerator at 4°C. Nutrient agar is selective but classified as general purpose because it allows the growth of many different non-fastidious species such as the bacteria used for this study. To derive the bacterial count using the McFarland turbidity test would have been better but was not used because of the cost implication. However, future studies would consider using this method.

Antimicrobial activity test of plant extracts

The measurement of zone of inhibition of the selected bacteria by the extracts (hot water and methanol) of *A. aureum* leaves was achieved by following the disc diffusion method illustrated by Bauer et al. (1966) and Barry et al. (1979). After sterilization of Mueller-Hinton Sensitivity Agar (MHA) medium, it was poured into sterile Petri dishes and allowed to solidify. Homogeneous day-old bacterial suspensions of the selected bacteria prepared in test tubes with sterile nutrient broth were used. With the aid of sterile cotton swab, each bacterial culture was inoculated separately on the surface of dry, sterile, solid MHA under aseptic conditions. Then the impregnated discs with 20 μl/disc of the various concentrations made from the two extracts were placed on the surface of MHA plates inoculated with a microbial culture. Each plate contained triplicate disc of each of the concentrations and standard antibiotic discs of chloramphenicol, and gentamycin which served as positive control (Figure 1). After 24 h of incubation, the plates were observed for the zone of inhibition and the diameter of the inhibition zone was measured in millimetre. There were four bacterial species (n=4) and five extracted concentrations (n=5) making a total of 20 replicates. Therefore, the number of replicates for both ethanol and water solvents were 40 (that is, n = 2×20).

Statistical analysis

An analysis of variance (ANOVA) was done (Logan, 2010) to determine whether there was any significant difference in the growth of microbes in diameter on different concentrations of *A. aureum* following the example of Quinn and Keough (2002). A Tukey HSD test was done to determine where the significant difference lies (Logan, 2010). Logarithmic transformation of the data was performed to meet assumptions of normality and homoscedasticity (Logan, 2010). Similarly, a post-hoc Tukey's HSD test was done to investigate pair wise mean differences between groups. All analyses were performed in *R* statistical environment, 3.0.1 (R Development Core Team, 2014).

RESULTS

Effect of different concentrations of *A. aureum* on microbial zone of inhibition

The ANOVA result indicates that there is a significant

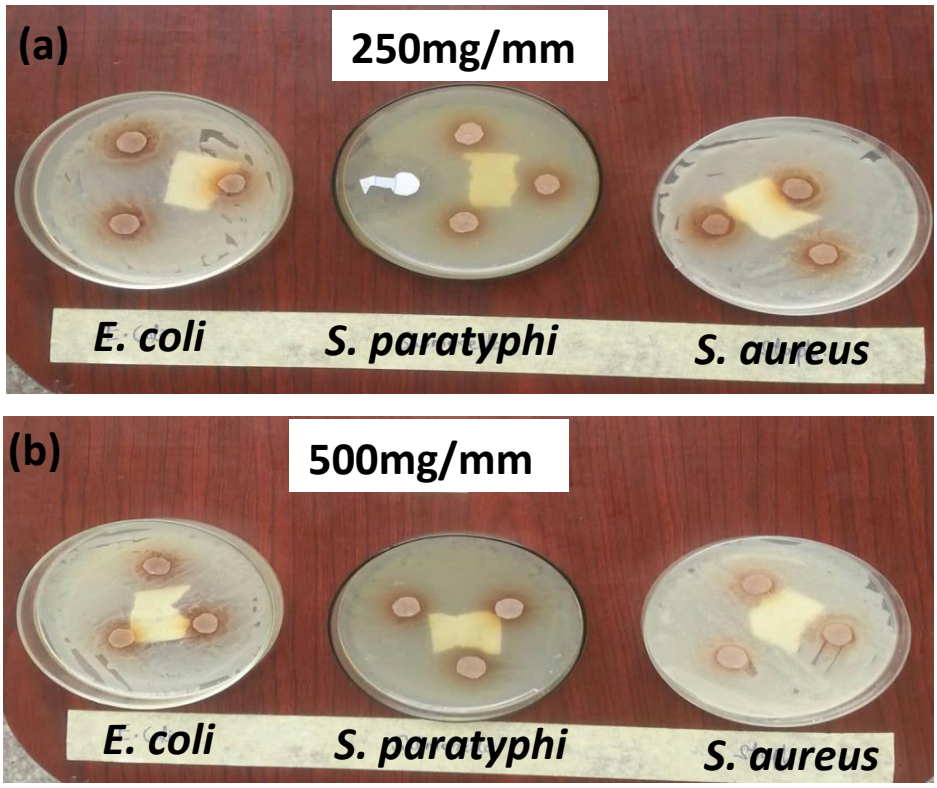

Figure 1. Culture plates with zones of inhibition (diameter in millimetre) for concentrations of 250 mg/mm and 500 mg/ml with hot water used as solvent.

difference in the zone of inhibition of the microbes on the different concentrations of *A. aureum* ($F_{4, 100}$ = 4.02, P = 0.01) (Figure 2 and Table 1). Similarly, the Tukey HSD test shows that the greatest difference in the zone of inhibition was observed in *A. aureum* concentrations of 62.5 and 500 and 62.5 and 250 mg/ml, respectively at P < 0.05. *A. aureum* concentrations of 500 and 250 mg/mm had the largest zones of inhibition and thus, the greatest inhibitory effect on all bacterial growth (Figure 1). Among the four bacterial species used for the study, *A. aureum* extract had the highest inhibitory effect on *E. coli* and *S. aureus* (Table 1) whereas the lowest inhibitory effect was recorded on *S. paratyphi*.

The result shows that higher concentration of *A. aureum* was more potent in inhibiting the growth of the microbes. There is thus a reduction in diameter as the concentration of *A. aureum* extracts decreases. It shows that *A. aureum* concentration of 500 mg/ml has the average highest zone of inhibition between the concentrations.

Secondly, the ANOVA result indicates that there is a significant difference in the zone of inhibition (diameter) between the microbial species ($F_{3, 101}$ = 12.08, P = 0.001). Similarly, the Tukey HSD test shows that the greatest difference was observed between *S. paratyphi* and *E. coli* at P<0.05 being species that has the lowest and greatest impact of the *A. aureum* extract, respectively.

The effect of *A. aureum* extractive solvents on microbial zone of inhibition

The ANOVA result indicates that there is a significant difference in microbial zone of inhibition (diameter) between the two extracts (that is, methanol and water) used ($F_{1, 103}$ = 6.43, P = 0.01) (Figure 3). The concentrations of all *A. aureum* extracts prepared with methanol has higher zone of inhibition than those prepared with hot water (Table 2). Furthermore, the concentrations with the highest zone of inhibition were the methanol extracts of 500 mg/mm (11.08±0.58 mm) and 250 mg/mm (11.33±0.48 mm), whereas the least zone of inhibition was recorded for water extract for concentration 62.5 mg/mm (3.25±1.18 mm). The zones of inhibition for extracts of 31.25 to 125 mg/ml are statistically significant as compared to 250 and 500 mg/ml that are not statistically different as shown by the error bars (Figure 3).

DISCUSSION

Studies show that *A. aureum* contains flavonoids, phthalates, sterols and terpenoids, and has some anti-bacterial properties because of its strong effect on *E. coli.* However, this is contrary to a study by Lai et al. (2009)

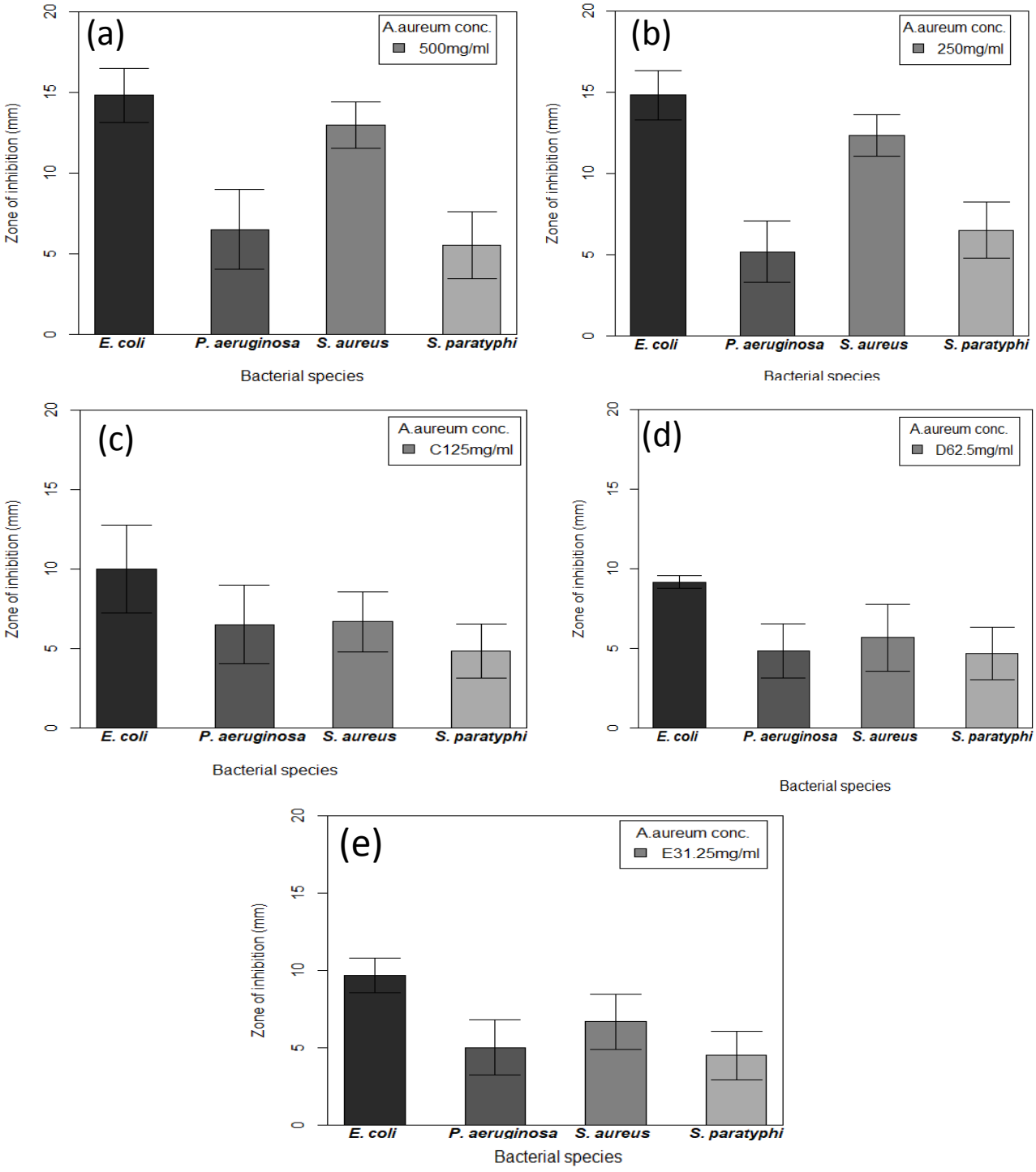

Figure 2. Zone of inhibition (mm) of bacteria species on different concentrations of *A. aureum* (a) 500 mg/ml, (b) 250 mg/ml, (c) 125 mg/ml, (d)62.5 mg/ml and (e) 31.25 mg/ml.

who showed that *A. aureum* has no anti-bacterial properties on some bacterial species such as *Micrococcus luteus*, *Bacillus cereus*, and *S. aureus*. The same study shows that the methanol extracts inhibited *Serratia marcescens* and *E. coli*. Furthermore, *A. aureum* extract has amino group and other chemicals that allows it to penetrate the cell walls of Gram-negative bacteria (Lode, 2001) making it a therapeutic agent (Nugraha et al., 2020). It is also a protein synthesis inhibitor and it inhibits enzyme reactions, and thus, controls the growth

Table 1. Mean zones of inhibition (mm) by bacterial species at different concentrations of *A. aureum* extracts ± 1 SE.

Bacterial species	Concentrations of *A. aureum* extracts (mg/ml)					P-value
	500 mm	250 mm	125 mm	62.5 mm	31.25 mm	
E. coli	14.83±1.68	14.83±1.52	10.00±2.76	9.17±0.40	9.67±1.12	0.01
S. paratyphi	5.50±2.08	6.5±1.75	4.8±1.72	4.7±1.65	4.5±1.56	
S. aureus	13.00±1.44	12.33±1.28	6.67±1.87	5.67±2.11	6.67±1.80	
P. aeruginosa	6.50±2.47	5.17±1.91	6.50±2.47	4.83±1.72	5.00±1.79	

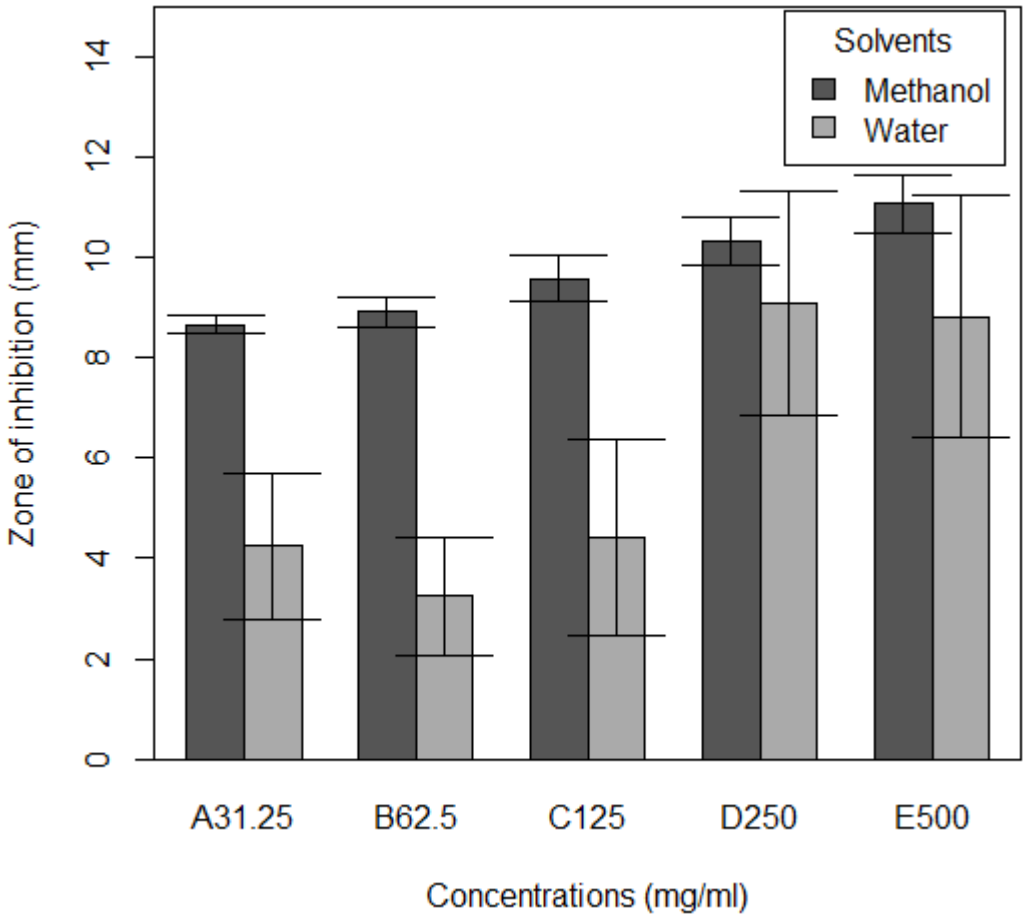

Figure 3. Effect of different solvents (that is, water and methanol) on bacterial growth on different concentration of *A. aureum*. Dark bars represent methanol while light bars represent water.

of microorganisms. *E. coli* is a Gram negative bacteria and the inhibition of its growth in the agar plates containing different concentrations of *A. aureum* is made possible because *A. aureum* can penetrate its cell wall and control its growth. Other studies used different species of *E. coli* to test the effectiveness of different antibiotics (Hernández-Porras et al., 2004). Some pathogenic bacteria also develop resistance against antibiotics, which makes them spread fast and difficult to control (May et al., 2009). Out of all the bacterial species *E. coli*, which is a Gram-negative bacteria had the highest growth inhibition by *A. aureum* extracts as compared to the Gram-positive bacteria (Figure 2 and Table 1). Martin (1995) reported that Gram negative bacteria are more resistant than Gram positive, but in this study the Gram-negative *E. coli* was the least resistant than the Gram positive. This can be attributed to the thin peptidoglycan layer of the cell wall of the Gram-negative bacteria. This layer as it grows eventually give way to a softer lipopolysaccharide outer membrane layer (Martin, 1995;

Table 2. Mean zone of inhibition of different extracts treated with two solvents, that is, water and methanol ± 1 SE.

Reagent	Concentrations of *A. aureum* extracts (mg/ml)					P-value
	500 mm	250 mm	125 mm	62.5 mm	31.25 mm	
Methanol	11.08±0.58	11.33±0.48	9.58±0.45	8.92±0.31	8.67±0.19	0.01
Water	8.83±2.42	9.08±2.24	4.42±1.95	3.25±1.18	4.25±1.46	

Guardabassi and Courvalin, 2001) that is easily penetrated, and its growth inhibited by the *A. aureum* extracts. Other studies have shown that the leaves of *A. aureum* has pharmacological properties, and contain anti-cancer, anti-microbial and anti-inflammatory agents that can be used to manufacture drugs (Badhsheeba and Vadivel, 2020; Ningsih et al., 2019). The lipopolysaccharide layer of the Gram-negative bacteria confers lower permeability than the Gram-positive bacteria (Arthur et al., 1996). Generally, antimicrobial agents attack basically three aspects of bacteria, namely, cell wall, protein, and nucleic acid biosynthesis. The effect of *A. aureum* on *E. coli* is because it acts on a single species as compared to extracts of other mangrove species that acts on many species called multiple spectrum effect (Helmerhorst et al., 1997). The current study also revealed that the higher the concentration of *A. aureum* extract the more effective it is in controlling bacteria, especially for *E. coli* and *S. aureus* (Figure 2). Higher concentrations of antibiotics have higher killing ability but must be checked regularly to ensure that it does not have adverse effect on humans when used as drugs to control diseases.

The methanol leaf extract of *A. aureum* had greater effect than the hot water extract, which is in line with studies done by Khan et al. (2013) who showed that ethanol leaf extract of *A. aureum* exhibited significant free radical scavenging activity with IC50 value of 42 µg/mL with higher effect on bacteria species. Similarly, Arora et al. (2019) showed that *A. aureum* has antimicrobial metabolites. Furthermore, other studies show that the methanol extract of *A. aureum* shows anti-cancer properties (Uddin et al., 2011). Results of previous and present studies show that there is high possibility of using *A. aureum* to manufacture medicinal products if the pure active ingredients of the extracts are tapped.

CONCLUSION AND RECOMMENDATION

This study shows that the mangrove species *A. aureum* has antibacterial properties, which means it can be used to produce antibiotics. It further revealed that *A. aureum* is more effective in eliminating *E. coli*, which is a major causative agent of food poisoning and many other diseases such as diarrhea urinary tract infections pneumonia, and respiratory illnesses prevalent in the Niger Delta region. There is thus a possibility of producing drugs with extracts of *A. aureum* if the extraction processes are effectively done to derive the undiluted active antibiotic ingredient. For example, micro-wave assisted extraction method (Mandal et al., 2007) can be used to extract better concentration that will give better antibiotic effect. For this study, the leaves alone were used for the extraction, but future studies will consider the use of other parts of the mangrove, such as root, bark, stem, and seeds to compare the parts with the best antibacterial properties. Finally, it was revealed that higher concentrates of *A. aureum* extracts (250 and 500 mg/ml) were more effective in controlling bacterial growth. Future studies will compare even higher concentration to determine the best threshold that will eliminate more bacterial species and in general other microbes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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